

Shared developmental roles and transcriptional control of autophagy and apoptosis in *Caenorhabditis elegans*

PhD thesis

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1 Introduction

Programmed cell death (PCD) is essential in animal development and tissue homeostasis. There are three major types of PCD: apoptosis (type I PCD), autophagy (type II PCD) and necrosis (type III PCD), although necrosis is not always considered as programmed cell death. PCD functions during animal development to eliminate certain cells, tissues or complete organs, for example larval structures in insects. It helps in the formation of developing tissues, regulates cell number and eliminates dangerous cells. Abnormal function of PCD can lead to many diseases in human, including neurodegeneration, immunological and developmental disorders or cancer.

Apoptosis is the most studied and best understood form of PCD. This process exists in all multicellular organisms. The function of apoptosis is necessary not only during different phases of development but also during adulthood. Four major roles are ascribed to apoptosis: 1) driving morphogenesis, 2) deletion of unnecessary tissues or complete organs, 3) regulation of cell number, and 4) elimination of certain cells. Interestingly, the absence of this cellular process compared to its importance causes relatively minor malformations in the development of *C. elegans*, in comparison with higher organisms such as mouse or human.

The core apoptotic machinery was first described in *C. elegans*. The cell number of the worm is fixed and the pattern of somatic cells dying by apoptosis during development is highly reproducible. The mechanism of apoptosis is divided into three steps: i) initiation and execution, ii) DNA fragmentation, iii) cell corpse engulfment.

According to our knowledge, in *C. elegans*, apoptosis is modulated mostly cell-autonomously. Hence, the activity of *egl-1* is regulated at the transcriptional level. The neuro-secretory motoneuron (NSM) cells differentiate into serotonergic neurons, while their sisters, the NSM sister cells, undergo programmed cell death during embryogenesis. A factor called cell death specification-1 (CES-1) blocks *egl-1* expression, thereby promoting survival. In normal conditions the expression of *ces-1* is repressed by the basic leucine zipper (bZIP) transcription factor CES-2 leading to death of NSM sister cells.

Autophagy is a highly regulated self-degradation process of all eukaryotic cells. Its mechanism is evolutionarily conserved from yeast to mammals. The basal, constitutive level of autophagy plays an important role in cellular homeostasis through the elimination of

damaged/old organelles, as well as the turnover of long-lived proteins and protein aggregates. Thus it ensures quality control of essential cellular components. Furthermore, when cells are under environmental stress, such as nutrient starvation, hypoxia, oxidative stress, pathogen infection or irradiation, the level of autophagy can be dramatically elevated as a cytoprotective response, resulting in adaptation and survival. On the other hand, excessive autophagy can lead to cell death under certain circumstances.

During autophagy parts of the cytoplasm, containing macromolecules or cellular organelles are sequestered into a double-membrane vesicle, termed autophagosome. Autophagosome is then fused with a lysosome, thereby forming a structure called autolysosome, where the separated cytoplasmic content is degraded by lysosomal hydrolases. The resulting end-products of autophagic breakdown are transported back into the cytosol through membrane permeases for synthetic processes. The mechanism of autophagy can be divided into four steps: i) induction, ii) vesicle nucleation, iii) vesicle elongation and closing, iv) maturation of autophagosome and fusion with the lysosome. Genes encode proteins participating in different steps of autophagy are well characterized.

In *C. elegans* autophagy is involved in the elimination of P granules from somatic cells, in the degradation of the paternal mitochondria and in dauer development.

2 Aims

We were interested in the interplay between autophagy and apoptosis during embryonic and postembryonic development of *C. elegans*. Apoptosis-deficient single mutant nematodes are viable, fertile, and their morphology and behavior do not differ significantly from that of the wild type. Single loss-of-function mutants for several autophagy genes are also viable and fertile. To answer the question, how apoptosis and autophagy are related to each other during development, first we intended to create double mutant animals, lacking the function of both autophagy and apoptosis. Then we planned to examine their phenotypes compared to wild type and the corresponding single mutants. Furthermore we wished to measure the level of apoptosis in different autophagic mutant backgrounds, and *vice versa*, the rate of autophagy in animals lacking apoptosis.

Next, we planned to search for transcription factors that may act as regulators of both autophagy and apoptosis. We tried to find conserved transcription factor binding sites of known apoptosis regulators in the 5' regulatory region of autophagy genes using

bioinformatic methods. We wished to validate our *in silico* results applying an *in vitro* technique called electrophoretic mobility shift assay or EMSA. Finally, we planned to generate transgenic *C. elegans* strains carrying integrated copy of reporter constructs that contain 1) the analysed regulatory sequences and 2) their mutant counterparts lacking the potential binding sites.

3 Materials and methods

1. Maintenance of *C. elegans* strains

Worms were grown in termostates at 15-20 °C on plastic Petri dishes containing nematode growth medium (NGM) agar. A bacterial lawn of *Escherichia coli* OP50 strain served as food source for nematodes.

Sexual dimorphism is characteristic for *C. elegans*. Propagation of self-fertilizing hermaphrodites allows the maintenance of invariant genetic lines, while crossing hermaphrodites with males permits the establishment of different genetic combinations.

2. RNAi treatment

It was found that feeding worms on bacteria engineered to produce dsRNA specific for a gene of interest induces a robust RNAi response. During this method, worms are fed by special *E. coli* bacteria (i.e. HT115 is an *E. coli* strain lacking RNaseIII), which carry the feeding construct containing a part of the gene of interest.

3. Determining lethality and brood size

For measuring embryonic and larval lethality about twenty adult hermaphrodites were transferred to a clean NGM plate to lay eggs for two hours at room temperature (synchronisation), then they were removed and the exact egg number was counted. After 24 and 48 hours both the number of dead eggs and larvae and also the amount of the survivors were determined. For measuring the brood size, 10 adult worms were put on separated plates. All the animals were transferred on a new plate every day, and the eggs layed from the previous 24 hours were counted on each plate. This was repeated until the animals were fertile. Finally, the brood size of all hermaphrodites was summarized.

4. Light and fluorescence microscopy

For taking high magnification images an Olympus BX51 light microscope was used. Using Differential Interference Contrast (DIC) optics high quality Nomarsky images could be

obtained. For fluorescent images the light source was a mercury arc lamp and filters designed for detecting the desired fluorescent markers were used.

5. Measuring the level of apoptosis

Two different methods were used to determine the level of apoptosis in the developing embryos: 1) counting the number of apoptotic corpses by DIC optics and 2) the TUNEL assay. During the first technique images were taken from comma stage embryos at different focal planes. Later the exact number of cell corpses was counted in each focal plane. TUNEL staining is a special method developed to detect and quantify apoptotic cell death at the single-cell level in different tissues. TUNEL reaction fluorescently labels the free 3'-OH termini, generated during apoptosis.

6. EMSA (electrophoretic mobility shift assay)

Electrophoretic mobility shift assay (EMSA) is an important technique for determining sequence-specific protein-DNA interactions. During EMSA a mix of labeled oligonucleotides containing the hypothesized binding sites and the potential DNA binding protein is loaded on non-denaturing polyacrylamide gel. The DNA-protein complex migrates more slowly than the free DNA fragments during electrophoresis.

7. Cloning of reporter constructs

A genomic fragment containing the 5' regulatory region and the first two exons of *bec-1* was amplified. *bec-1::gfp* construct was prepared by cloning the BamHI/HindIII digested PCR product into pPD95.75 vector. A QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used to generate the mutated *bZipΔ_bec-1::gfp* reporter construct.

8. Generation of transgenic animals (microparticle bombardment)

The linearized DNA construct of interest, together with the linearized positive selection marker containing a full length wild type *unc-119* rescuing fragment, was bound to gold micro particles. Then the prepared animals were shot with the gold particles covered with DNA by a BioRad PDS-1000/He high pressure vacuum system (gene gun). Non-paralyzed animals were selected and integrated homozygous transgenic lines were generated.

4 Results

1) Autophagy and apoptosis are redundantly required for *C. elegans* development

- a) Most of the *atg-18(gk378)*, *ced-4(n1162)*, *ced-4(n2273)* and *ced-3(n717)* single mutants are viable and fertile. *atg-18(gk378)/+; ced-4(n1162)*, *atg-18(gk378)/+; ced-*

4(*n2273*) and *atg-18(gk378)/+; ced-3(n717)* heterozygous hermaphrodites produced viable homozygous double mutant F1 progenies. However, F2 descendants of *atg-18(gk378); ced-4(n1162)* showed a full penetrant embryonic lethality. Additionally F2 progeny of *atg-18(gk378); ced-4(n2273)* and *atg-18(gk378); ced-3(n717)* showed 36% and 47% embryonic lethality, respectively. Most of the survivors died at the L1 larval stage and only 1-2% of the animals reached the adulthood.

- b) In comparison with the wild-type embryos *atg-18(gk378); ced-4(n1162)* double mutant embryos exhibit severe defects, such as elongation deficiency, body shape malformations, the presence of extraembryonic “shed cells” and necrotic-like holes. The organs and the tissues start to develop, e.g., the pharynx and the gut precursor cells.
- c) According to my results, 14% and 4% of the *unc-51(e369)* single mutants died during embryonic and larval development, respectively. Compared to these data, embryonic and larval lethality of *unc-51(e369); ced-4(e1162)* double mutants was increased to 27% and 39%, respectively. Homozygous *unc-51(e1189)* single mutants displayed 15% embryonic and 15% larval lethality. In turn, 39% of *unc-51(e1189); ced-4(e1162)* double mutants died as embryos and 45% of them showed larval lethality.
- d) *unc-51(-); ced-4(-)* double mutant worms, which died either during embryogenesis or during postembryonic development, showed serious morphological defects. Although different tissues and organs started to develop, the correct, healthy shape of the soma could not be formed. Invaginations were observed both in embryos and in hatched larvae. Positions of some organs in the double mutant larvae were also abnormal.
- e) Although *atg-7(tm2976)* and *lgg-1(tm3489)* homozygous single mutants, progeny of heterozygous animals, were viable, the F2 generation displayed full penetrant embryonic and early larval lethality. The F1 generation of *atg-7(tm2976); ced-4(n1162)* and *lgg-1(tm3489); ced-4(n1162)* double mutants were also viable, but all of their offsprings died already during embryogenesis.
- f) The phenotype of *atg-7(tm2976); ced-4(n1162)* and *lgg-1(tm3489); ced-4(n1162)* double mutant embryos were similar to that of *atg-18(gk378); ced-4(n1162)* double mutants.

- g) Silencing of *bec-1* by RNAi resulted in a fertile F1 generation, with only 10% embryonic lethality. When *ced-4(n2273)* and *ced-3(n717)* mutants were treated with *bec-1* dsRNA, half of the F1 progeny died as embryos, the other half reached the adulthood, but became sterile.
 - h) I found that the average brood size of *atg-18(gk378)*, *ced-4(n1162)* and *ced-4(n2273)* single mutant animals was reduced by 15-20%, compared to the wild type. The *ced-3(n717)* mutation seemed not to influence the brood size of the worms. *atg-18(gk378); ced-4(n1162)*, *atg-18(gk378); ced-4(n2273)* and *atg-18(gk378); ced-3(n717)* double mutants produced approximately 53, 35 and 27% less embryos, respectively, compared to the wild type.
 - i) We counted the number of apoptotic corpses in comma stage embryos, and found an average of 1.5 in wild-type animals. At the same time, the average number of the observed apoptotic events in embryos lacking *lgg-1* activity is 4. Using TUNEL staining, we counted an average 1.3 fluorescent signs indicating apoptotic events in wild-type embryos and it was more than two times higher in *atg-18(gk378)* and *unc-51(e369)* mutants.
 - j) I checked *lgg-1* expression in *ced-4(n1162)* mutant background at different developmental stages, but I could not detect any difference in the pattern or expression level of the transgene compared to wild type
- 2) Autophagy and apoptosis are transcriptionally co-regulated in *C. elegans***
- a) Using bioinformatics, we found a conserved binding site for the CES-2/ATF-2 transcription factors approximately 400 base pairs upstream of the translational initiation site of the *bec-1* gene. After analysing the corresponding *C. remanei* and *C. briggsae* genomic regions, we found that they display only minor changes in these sites, compared to that of *C. elegans*.
 - b) ATF-2 was able to bind to a putative binding site of *bec-1* *in vitro*, while it could not bind to it when the target sequence was previously mutated. CES-2 could also bind to the oligonucleotides if they contained the candidate site, and there was no interaction in its absence.
 - c) At the embryonic comma stage, and at the L2 and L4 larval stages examined, we could observe a significant increase in the expression of the *bZipΔ_{bec-1}::gfp* construct, as compared to *bec-1::gfp*.

5 Discussion

1) Autophagy and apoptosis are necessary together for the viability of *C. elegans*

- a) The survival of the F1 generation of the *atg-7(tm2976)*, *lgg-1(tm3489)* single mutants and autophagy-apoptosis homozygous double mutant animals, in contrast to the lethality of the F2 progeny, can be explained by maternal contribution of the examined autophagy genes.
- b) From these data, it can be concluded that although single mutants for autophagy or apoptosis produce healthy, wild-type-like offsprings, these cellular processes do function during embryonic development of *C. elegans*. Maternal effect of autophagy genes further supports the idea about the importance of autophagy during embryogenesis.
- c) The penetrance and expressivity of the observed phenotypes depended on which autophagy gene was inactivated and also on the characteristics of the mutation. Additionally, the applied technique whether the corresponding gene was silenced by RNAi or inactivated by a mutation also had an influence on the resulting phenotypes. It was also important which apoptosis gene was knocked out and whether the used mutation was hypomorphic or amorphic.
- d) At least one of the two mechanisms should be present for normal embryogenesis.

2) Simultaneous inactivation of autophagy and apoptosis causes severe morphological malformations during embryogenesis

- a) Most of the autophagy-apoptosis defective double mutants did not display the special shape of a lima bean embryo which is a result of the ventral enclosure. I suggest that this step, which is strongly necessary for the elongation, is affected. There are epidermal leading cells which drive the ventral enclosure and maybe these cells are missing or their movement is blocked in the analyzed worms lacking both autophagy and apoptosis.
- b) When both autophagy and apoptosis are compromised, starving embryonic cells might undergo cell death, probably by necrosis. The observed necrotic-like vacuoles in autophagy-apoptosis defective double mutant embryos support this idea. A still not identified regulatory link between the three cell death pathways might exist, and in nematodes lacking both autophagy and apoptosis, necrosis might be activated.

- c) The presence of extra embryonic “shed cells” could mean that the embryo lacking the basic cell elimination mechanism tries to dispose of the superfluous cells.
 - d) In the analyzed autophagy-apoptosis double mutant embryos complete organs seemed to appear, thus differentiation of different cell types may not be affected in the absence of both processes. In summary, the two mechanisms are essential together for the normal development of *C. elegans*
- 3) Autophagy and apoptosis are redundantly required for the embryonic development of *C. elegans***
- a) In autophagy deficient mutants apoptotic activity was increased in comparison with the wild type. I found two possible explanations for these results: 1) autophagy acts in certain cells to protect them from undergoing apoptosis, or 2) in the absence of autophagy the engulfment process is impaired.
 - b) I could observe no difference between *gfp::lgg-1* and *gfp::lgg-1; ced-4(n1162)* strains in the expression pattern of *lgg-1*. It is possible that the used system is not sensitive enough to detect the difference in this punctuated pattern.
 - c) The synthetic lethal phenotype of autophagy-apoptosis defective double knockouts and the fact that apoptotic activity is elevated in autophagic mutant background suggest that the two mechanisms act redundantly during embryonic development of this model organism.
- 4) Autophagy and apoptosis together have an influence on the fertility of *C. elegans***
- The sterility phenotype of *bec-1(RNAi); ced-4(n2273)* and *bec-1(RNAi); ced-3(n717)* animals together with the synthetic brood size reduction phenotype of the autophagy-apoptosis double mutants suggest that autophagy and apoptosis may participate in germ cell production. In the absence of apoptosis maybe autophagy serves as a backup mechanism to ensure nutrient supply for oocytes.
- 5) The role of *bec-1* in the regulation of autophagy and apoptosis**
- a) We found a sequence motif of CES-2 and ATF-2 bZip-like transcription factors in the promoter of *bec-1*. This element is conserved among several *Caenorhabditis* species, thus the regulation of *bec-1* and its orthologs mediated via this sequence are phylogenetically conserved.

- b) In the gel retardation assay neither of the two transcription factors was shown to interact with the binding site when it was mutated, thus the protein-DNA interactions take place specifically.
- c) The expression level of *bZipΔ_bec-1::gfp* reporter construct was elevated in several tissues in embryos, as well as in L1 and L4 larvae, in comparison with that of *bec-1::gfp*. In embryos, *bec-1* can be modulated either by ATF-2 or CES-2, or maybe by both of them, since they are present in this developmental stage and it is known that the bZip-like transcription factors can function as homodimers and heterodimers as well. In larvae, only *atf-2* is expressed, thus in postembryonic developmental stages this bZip-like transcription factor should regulate *bec-1*. Thus autophagy and apoptosis share regulation by common transcription factors.
- d) According to my model, the level of ATF-2, and/or maybe CES-2, is low in those cells of the embryos which are fated to live. Here, *bec-1* can be expressed, supporting autophagy and inhibiting apoptosis. In cells destined to die, high amount of ATF-2, and/or maybe CES-2 blocks *bec-1* which results in low levels of autophagic activity and the induction of apoptosis.

6 Publications related to my dissertation

- Erdélyi P, Borsos E, Takács-Vellai K, Kovács T, Kovács AL, Sigmond T, Hargitai B, Pásztor L, Sengupta T, Dengg M, Pécsi I, Tóth J, Nilsen H, Vértessy BG, Vellai T (2011). Shared developmental roles and transcriptional control of autophagy and apoptosis in *Caenorhabditis elegans*. *J Cell Sci.* **124**(Pt 9):1510-8.
- Borsos E, Erdélyi P, Vellai T (2011). Autophagy and apoptosis are redundantly required for *C. elegans* embryogenesis. *Autophagy* **7**(5):557-9.
- Várkuti BH, Yang Z, Kintses B, Erdélyi P, Bárdos-Nagy I, Kovács AL, Hári P, Kellermayer M, Vellai T, Málnási-Csizmadia A (2012). A novel actin binding site of myosin required for effective muscle contraction. *Nat. Struct. Mol. Biol.* **19**(3):299-306.